View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Digital.CSIC

β-glucan administration enhances disease resistance and some innate immune responses in zebrafish (*Danio rerio)*

Iván Rodríguez, Rubén Chamorro, Beatriz Novoa and Antonio Figueras*

Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (CSIC), Eduardo Cabello 6, Vigo, Spain

*Corresponding author

Submitted to Fish and Shellfish Immunology as a Short Communication

Revised version

Abstract

The present study was conducted to investigate the effect of β-glucan (derived from *Sacharomyces cerevisiae)* on the immune response and its protection against an infection of the bacterial pathogen *Aeromonas hydrophila* in zebrafish (*Danio rerio).* Zebrafish received β-glucan by intraperitoneal injection at three different concentrations (5, 2 and 0.5 mg/ml) at 6, 4 and 2 days prior the challenge. On challenge day the control and β-glucan pretreated zebrafish were intraperitoneally injected with *A. hydrophila* and mortality was recorded for 4 days. Intraperitoneal injection of 5 mg/ml of β-glucan significantly reduced the mortality. A single injection of 5 mg/ml of β-glucan 6 days before challenge also enhanced significantly the survival against the infection. The treatment with β-glucan increased the myelomonocytic cells population from kidney at 6 hours postchallenge with *A. hydrophila.* Moreover it enhanced the ability of kidney cells to kill *A. hydrophila.* β-glucan did not affect the expression of TNFα, IL1β but it seemed to modulate the IFNγ and chemoquines expression in kidney.

Keywords: Zebrafish; β-glucan; Immune response; Disease resistance; Intraperitoneal injection; *Aeromonas hydrophila*

Introduction

β-glucan is a heterogeneous group of glucose polymers, consisting of a backbone of β-(1→3)-linked β-D-glucopyranosyl units with β-(1→6)-linked side chains of varying length and distribution. These polysaccharides are major cell wall structural components in fungi and are also found in plants and some bacteria. β-glucan has been shown to be immunostimulant and to posses an array of beneficial properties, including enhancing protection against infections [1,2], tumour development [3,4] and sepsis [5,6].

The effect of β-glucan has been attributed to its binding to several receptors on leukocytes resulting in the stimulation of immune responses, such as bacteria killing activity [2], modulation of cytokine production [7,8] and survival promotion at the cell, organ and whole animal levels [8,9].

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems such as freshwater, coastal water, and sewage [10]. They are increasingly being reported, especially *Aeromonas hydrophila*, which is responsible for haemorrhagic septicemia, a disease affecting a wide variety of freshwater fish species and occasionally marine fish [11-15]. Furthermore, the bacterium is an emerging human pathogen that causes a variety of diseases, most commonly gastroenteritis, wound infections and septicemia, in children and adults [16,17].

Several extracellular toxins and enzymes that may be associated with the virulence of *A. hydrophila* such as hemolysins, cytotoxins, enterotoxins and proteases [18,19] have been described. These virulence factors induce acute inflammatory responses [20,21] enhancing the expression of genes encoding proinflammatory cytokines [22].

The aim of this work was to investigate the effect of intraperitoneal injection of β-glucan on zebrafish, *Danio rerio*, experimentally infected with *A. hydrophila*. To study this effect we examined the survival outcome in β-glucan treated infected zebrafish, the

70 percentage of myelomonocytic cells from kidney cells, the bacteria killing ability and
71 the expression of proinflammatory cytokines.
72

73 Materials and Methods

74 Care and feeding of zebrafish followed established protocols [23] (also see
75 http://zfin.org/zf_info/zfbook/zfbk.html). Zebrafish wild type adults (1-1.5 g ; 4-5 cm)
76 were anesthetized with MS-222 (Tricaine methanesulfonate, Argent Chemical
77 Laboratories, USA). Euthanasia of zebrafish was obtained by an anaesthetic overdose.
78 The bacteria, isolated from zebrafish [24], were grown on tryptic soy agar (TSA) plates
79 for 24 h at RT and, after incubation, cells were recovered in sterile phosphate buffered
80 saline (PBS). Quantitation of logarithmic cultures was performed by spectrophotometry
81 and plating dilutions of the culture on TSA.
82 For the challenge study, the ß glucan injections were performed as described by Selvaraj
83 et al. [2] Four groups (2 replicates of 12 zebrafish/group) were inoculated with 10 µl of
84 5, 2 or 0.5 mg/ml of β-glucan using a 0.5 ml [0.3 mm (30G) x 8 mm] syringe; 6, 4 and 2
85 days prior the inoculation of 10 µl from *A. hydrophila* (10^8 cfu/ml). Controls were
86 injected with 10 µl of PBS. The mortality was recorded daily up to 4 days.
87 Another experiment was performed. Six groups (2 replicates of 12 zebrafish/group)
88 were pretreated by a single ip injection with 10 µl of 5 mg/ml of β-glucan or PBS at 2, 4
89 or 6 days prior to challenge.
90 To determine the changes in the percentage of myelomonocytic cells from kidney, 12
91 zebrafish were ip inoculated with 10 µl of β-glucan at a dose of 5 mg/ml and another 12
92 with PBS as was described above. On challenge day, 6 zebrafish from the β-glucan
93 group were ip inoculated with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml and the
94 remaining 6 zebrafish were mock injected with PBS. After 6 hours, the kidneys of

zebrafish of each group were removed aseptically and homogenised in two pools. Flow cytometry analysis of the myelomonocytic cells population was based on forward and side scatter on a FACScalibur flow cytometer (Beckton Dickinson) using previously reported settings for the myelomonocytic cells population [24].

The bacterial killing assay was performed according to Chen and Ainsworth [25]. Three zebrafish were inoculated ip with 10 µl of PBS and another group of 3 zebrafish with 10 µl of 5 mg/ml of β-glucan as indicated in the challenge experiment description. On day 7 after the first inoculation, the kidney cells were obtained and suspended in D-MEM:F12 containing 10% foetal bovine serum (FBS) at a concentration of $5x10^5$ phagocytes/ml. From this, 0.1 ml was taken and mixed with 0.1 ml of *A. hydrophila* ($5x10^6$ cfu/ml), mixed well and incubated for 2 hours with occasional shaking in a water bath at 28ºC. After 2h, 0.1 ml of the bacteria/kidney cells mixture was diluted on 9.9 ml of sterile distilled water to release living bacteria from phagocytes. This was serially diluted, plated on TSA agar plates, incubated overnight at RT and the number of colonies was counted.

A group of 72 zebrafish was ip inoculated with β-glucan at a dose of 50 µg/fish and another group of 72 animals with PBS to determine the tumor necrosis factor α (TNFα), interleukin 1 β (IL1β) and interferon γ (IFNγ) expression. The fish in each experimental treatment group (glucan or PBS) were redivided into two subgroups. Fish in a subgroup were challenged with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml, whereas fish in another subgroup were mock infected with PBS by ip injection. After 30 min, 2, 4 and 6h of challenge, the kidneys were sampled and kept in Trizol. Three pools (3 zebrafish/pool) for each treatment and sample time were prepared. Briefly, RNA extraction was performed using Trizol Reagent (Invitrogen). The reverse transcription

was performed with the SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer indications.

Quantitative PCR assays were performed using the 7300 Real Time PCR System (Applied Biosystems). cDNA amplification for β–actin, TNFα, IL1β and IFNγ was performed using specific primers described in [24] and the amplification of CXCL-C1c, CC-chemokine and IL8 was performed using specific primers designed by Primer 3 software [26]. Primer sequences are shown in Table 1. Each primer (0.5 μl with a concentration of 10 μM) was mixed with 12.5 μl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 μl. The standard cycling conditions were 95 º for 10 min, followed by 40 cycles of 95 º 15 s and 60 º for 1 min. The comparative CT method (2-ΔΔCT method) was used to determine the expression level of analyzed genes [27]. The expression of the candidate genes was normalized using β–actin as a housekeeping gene. Fold units were calculated dividing the normalized expression values of infected tissues by the normalized expression values of the controls.

Data were compared using the Student´s t-test. The results are expressed as mean ± standard deviation and differences were considered significant at *$P < 0.05$.; ** $P<0.01$.

Results and discussion

Our results indicate that the mortality due to infection with *A. hydrophila* was reduced by injecting different concentrations of β-glucan for three times at 6, 4 and 2 days prior to challenge. The protective effect of ip injection of β-glucan against several infections with pathogens has also been reported previously in different fish species [2, 28]. However the group of fish injected with 5 mg/ml of β-glucan was the only group that showed a significantly reduction of the mortality (figure 1A). A similar dose dependent response to the ip injection with β-glucan has been also reported [2].

144 The single inoculation of 5 mg/ml of β-glucan at different days, 6, 4 or 2 prior to
145 challenge, showed that it enhanced the protection against bacterial infection; although
146 the inoculation 6 days prior to challenge was the only one that reduced significantly the
147 mortality (figure 1B). Similar results have been reported for brook trout (*Salvelinus*
148 *fontinalis)* in a challenge with *Aeromonas salmonicida* [29].
149 The β-glucan pretreated zebrafish showed a percentage of myelomonocytic cells
150 significantly higher than the fish pretreated with PBS and although the infection with
151 bacteria reduced the population of myelomonocytic cells, the percentage of these cells
152 was still higher in the β-glucan pretreated zebrafish (figure 2A). These results are in
153 accordance with previous results [30] that showed that a derivative of glucan (PGG-
154 glucan) enhanced human myelopoiesis. In mice, both intravenous and ip glucan
155 injection resulted in increased bone marrow proliferation [31, 32]. On the other hand
156 Jorgensen et al. [33] observed that 3 weeks after ip injection of glucan in salmon did not
157 produce changes in mean values of head kidney macrophages in both glucan and saline
158 treated salmon although the number of neutrophils increased significantly in the head
159 kidney of the glucan treated salmon. Furthermore the myelomonocytic cells increase
160 may be also explained by a priming effect for chemotaxis in circulating neutrophils to
161 the kidney [34].
162 *A. hydrophila* was killed more efficiently by kidney cells of zebrafish inoculated with β-
163 glucan than zebrafish inoculated with PBS. Bacterial count was significantly reduced in
164 glucan injected fish compared with the PBS injected fish after 2 h of incubation with
165 kidney cells (figure 2B). This increase of bactericidal activity has been previously
166 reported in carp [2].
167 The mRNA expression levels of proinflammatory cytokines and chemokines were
168 determined by real time PCR in kidney of adult zebrafish pretreated with β-glucan or

169 PBS at days 1, 3 and 5 and subsequently inoculated with *A. hydrophila* or mock infected
170 with PBS at day 7 for 30 min, 2, 4 and 6 hours (figure 3).
171 TNFα expression levels in *Aeromonas* injected zebrafish showed an increase over
172 control levels at 4 hours post-inoculation (hpi), this increase was even higher when the
173 zebrafish were pretreated with β-glucan. The β-glucan pretreated zebrafish and PBS
174 mock infected showed a slight induction at 6 hpi.
175 IL1β expression levels had a 68 fold-induction at 2 hpi in PBS pretreated zebrafish and
176 infected with bacteria, they began to decline at 4 hpi. The zebrafish pretreated with β-
177 glucan and infected with bacteria presented the same kinetics as the PBS group,
178 however the peak at 2 hpi was lower. The effect of pretreatment with β-glucan in mock
179 infected zebrafish was a minimal expression increase at 30 min and 2 hpi.
180 The IFNγ expression levels of zebrafish pretreated with β-glucan and infected with
181 bacteria peaked at 4 hpi and they began to decline at 6 hpi, however the zebrafish
182 pretreated with PBS and infected with bacteria enhanced the expression levels at 6 hpi.
183 In the same way, the zebrafish pretreated with β-glucan and mock infected enhanced the
184 expression levels at 6 hpi.
185 Concerning chemokines expression, we observed that in the zebrafish pretreated with
186 PBS and infected with bacteria the expression was lower than the fish infected and
187 pretreated with β-glucan, except for IL8 at 2 hpi, although in this case the standard
188 deviation was high. In fish pretreated with β-glucan and mock infected a weak
189 expression of CXCL-C1c and CC chemokine was observed.
190 The β-glucan appears to be able to stimulate the production of proinflammatory
191 cytokines and chemokines, including TNFα, IL1β and IL8 [35]. Furthermore, this β-
192 glucan is thought to modulate cytokine production to secondary challenge, but there are

193 conflicting data as they have been shown to both prime and suppress these responses
194 [36, 37].
195 In disagreement with our findings, Sener at al. [6] and Toklu et al. [38] showed reduced
196 TNFα levels following administration of β-glucan on animal model of sepsis,
197 suggesting that the protective capacity of β-glucans may be due to modulation of the
198 cytokine profile. However Engstad et al. [36] found that β-glucan primed LPS
199 stimulation of TNFα and that the β-glucan itself was also able to induce a minor amount
200 of TNFα. On the other hand, two and three copies of TNFα have been cloned in rainbow
201 trout and carp, respectively, which have been named as TNF-1α, TNF-2α and TNF-3α
202 [39-41]. Furthermore, in zebrafish a novel TNF gene (TNF-N) has been identified that
203 is present upstream of TNFα gene in the same transcriptional orientation [42]. Although
204 there is a progress in cloning of TNF genes from various fish species, not many
205 functional data exists on fish TNF genes. Therefore, we must take into account that the
206 presence of different copies of TNF may influence our results.
207 Interestingly, the same situation occurs with IFNγ since in zebrafish two IFNγ genes
208 (IFNγ-1, IFNγ-2) have been identified and the expression analysis of these genes
209 suggests that they have an active role on immune responses in fish, where it was
210 showed that, while IFNγ-1 was expressed in normal tissues or treated with LPS and
211 Poly I:C, IFNγ-2 was expressed only after Poly I:C treatment [43]. In our case, we used
212 primers that amplified IFNγ-2 and it was observed that the expression increased earlier
213 in the zebrafish pretreated with β-glucan and infected with *A. hydrophila* than in PBS
214 pretreated fish. Furthermore, we found that β-glucan itself enhanced 18 fold the IFNγ
215 expression. Consequently, it seems that the β-glucan may modulate the expression of
216 IFNγ, which is a cytokine that is a strong activator of macrophages and the key of type 1
217 T helper (Th1) cell immune responses during infections with intracellular pathogens

[44]; moreover, it can up-regulate the capacity of monocyte-derived macrophages to phagocytose apoptotic cells [45]. Therefore, it seems that the immune cells could be more active and have a reaction time faster against a challenge. However, this increase of the expression of IFNγ does not seem sufficient to explain protection with β-glucan.

The chemokines are a family of cytokines that induce the migration of cells to sites of infection or injury in response to many stimulants, particularly proinflammatory cytokines such as IL1 and TNF [46]. In our case, we observed that the higher expression levels of chemokines mainly corresponded to fish pretreated with β-glucan and infected with *A. hydrophila*. CXCL-C1c and CC chemokine were found in a SSH performed in zebrafish infected with *A. hydrophila* for 1 hour and that enhanced their expression in the kidney [Rodríguez, unpublished results]. Therefore, they might play important roles in the response against the *A. hydrophila* infection. IL8 or CXCL8 are chemoattractive to basophils, cytokine-stimulated eosinophils and peripheral blood T lymphocytes in mammals [46]. In fish, it has been shown that trout CXCL8 expression is increased in head kidney macrophages following exposure to LPS and recombinant human TNFα [47]. Chemokines could be related with the increase in myelomonocytic cells through a priming effect for chemotaxis in circulating neutrophils to the kidney.

In summary, the literature on the effects of β-glucan on cytokine expression is inconsistent, probably reflecting a complex biological interplay as well as the use of different experimental systems and a variety of β-glucan preparations. Furthermore, in fish multiple isoforms of cytokines are present, possible due to a genome duplication event in bony fish [48]. These inconsistencies contribute to the enigma associated with the mechanisms by which β-glucan protects against some bacterial infections.

Acknowledgements

243 This work was supported by the project CSD2007-00002 “Aquagenomics”
244 funded by the program Consolider-Ingenio 2010 from the Spanish Ministerio de Ciencia
245 e Innovación. I. Rodríguez thanks the Consejo Superior de Investigaciones Científicas
246 for his I3P pre-doctoral contract.
247

References

[1] Guselle, NJ, Markham RJ and Speare DJ. Intraperitoneal administration of beta-1,3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*. J Fish Dis 2006; 29: 375-381.

[2] Selvaraj V, Sampath K and Sekar V. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol 2005; 19: 293-306.

[3] Cheung NK and Modak S. Oral (1-->3),(1-->4)-beta-D-glucan synergizes with antiganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res 2002; 8: 1217-1223.

[4] Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, Xing PX, Cheung NK and Ross GD, Mechanism by which orally administered beta-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. J Immunol 2004; 173: 797-806.

[5] Babayigit H, Kucuk C, Sozuer E, Yazici C, Kose K and Akgun H. Protective effect of beta-glucan on lung injury after cecal ligation and puncture in rats. Intensive Care Med 2005; 31: 865-870.

[6] Sener G, Toklu H, Ercan F and Erkanli G. Protective effect of beta-glucan against oxidative organ injury in a rat model of sepsis. Int Immunopharmacol 2005; 5: 1387-1396.

[7] Soltys J and Quinn MT. Modulation of endotoxin- and enterotoxin-induced cytokine release by in vivo treatment with beta-(1,6)-branched beta-(1,3)-glucan. Infect Immun 1999; 67: 244-252.

[8] Williams DL, Li C, Ha T, Ozment-Skelton T, Kalbfleisch JH, Preiszner J, Brooks L, Breuel K and Schweitzer JB. Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis. J Immunol 2004; 172: 449-456.

[9] Sandvik A, Wang YY, Morton HC, Aasen AO, Wang JE and Johansen FE. Oral and systemic administration of beta-glucan protects against lipopolysaccharide-induced shock and organ injury in rats. Clin Exp Immunol 2007; 148: 168-177.

[10] Monfort P and Baleux B. Dynamics of *Aeromonas hydrophila*, *Aeromonas sobria*, and *Aeromonas caviae* in a sewage treatment pond. Appl Environ Microbiol 1990; 56: 1999-2006.

[11] Candan A, Kücüker MA and Karatas S. Motile aeromonad septicemia in *Salmo salar* cultured in the black sea in Turkey. Bull Eur Ass Fish Pathol 1995; 15: 195-196.

[12] Kozinska A, Figueras MJ, Chacon MR and Soler L. Phenotypic characteristics and pathogenicity of Aeromonas genomospecies isolated from common carp (*Cyprinus carpio L.*). J Appl Microbiol 2002; 93: 1034-1041.

[13] Ogara WO, Mbuthia PG, Kaburia HFA, Sorum H, Kagunya DK, Nduthu DI and Colquhoun D. Motile aeromonads associated with rainbow trout (*Oncorhynchus mykiss)* mortlality in Kenia. Bull Eur Ass Fish Pathol 1998; 18: 7-9.

[14] Pullium JK, Dillehay DL and Webb S. High Mortality in Zebrafish (*Danio rerio)*. Contemp Top Lab Anim Sci 1999; 38: 80-83.

[15] Wang C and Silva JL. Prevalence and characteristics of Aeromonas species isolated from processed channel catfish. J Food Prot 1999; 62: 30-34.

[16] Galindo CL, Gutierrez C Jr and Chopra AK, Potential involvement of galectin-3 and SNAP23 in *Aeromonas hydrophila* cytotoxic enterotoxin-induced host cell apoptosis. Microb Pathog 2006; 40: 56-68.

[17] Janda JM and Abbott SL. Evolving concepts regarding the genus Aeromonas: an expanding Panorama of species, disease presentations, and unanswered questions. Clin Infect Dis 1998; 27: 332-344.

[18] Allan BJ and Stevenson RM. Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. Can J Microbiol 1981; 27: 1114-1122.

[19] Ljungh A, Wretlind B and Mollby R. Separation and characterization of enterotoxin and two haemolysins from *Aeromonas hydrophila*. Acta Pathol Microbiol Scand [B] 1981; 89: 387-397.

[20] Galindo CL, Fadl AA, Sha J, Gutierrez C Jr, Popov VL, Boldogh I, Aggarwal BB and Chopra AK. *Aeromonas hydrophila* cytotoxic enterotoxin activates mitogen-activated protein kinases and induces apoptosis in murine macrophages and human intestinal epithelial cells. J Biol Chem 2004; 279: 37597-37612.

[21] Galindo CL, Sha J, Ribardo DA, Fadl AA, Pillai L and Chopra AK, Identification of *Aeromonas hydrophila* cytotoxic enterotoxin-induced genes in macrophages using microarrays. J Biol Chem 2003; 278: 40198-40212.

[22] Chopra AK, Xu X, Ribardo D, Gonzalez M, Kuhl K, Peterson JW and Houston CW, The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect Immun 2000; 68: 2808-2818.

[23] Westerfield M. A guide for the laboratory use of zebrafish *(Danio rerio)*. University of Oregon Press: 2000.

[24] Rodríguez I, Figueras A and Novoa B. Immune response of zebrafish (*Danio rerio)* against a newly isolated bacterial pathogen *Aeromonas hydrophila*. Fish Shellfish Immunol 2008; 25: 239-249.

[25] Chen D and Ainsworth A. Glucan administration potentiates immune defence machanisms of channel catfish, *Ictalurus punctatus* Rafinesque. J Fish Dis 1992; 15: 295-304.

[26] Rozen S and Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000; 132: 365-386.

[27] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25 2001, pp. 402–408.

[28] Misra CK, Das BK, Mukherjee SC and Pattnaik P. Effect of multiple injections of beta-glucan on non-specific immune response and disease resistance in *Labeo rohita* fingerlings. Fish Shellfish Immunol 2006; 20: 305-319.

[29] Anderson DP, and Siwicki AK. Duration of protection against *Aeromonas salmonicida* in brook trout immunoestimulated with glucan or chitosan by injection or immersion. The Progressive Fish Culturist 1994; 56:258-261.

[30] Turnbull JL, Patchen ML and Scadden DT. The polysaccharide, PGG-glucan, enhances human myelopoiesis by direct action independent of and additive to early-acting cytokines. Acta Haematol 1999; 102: 66-71.

[31] Patchen ML and MacVittie TJ, Hemopoietic effects of intravenous soluble glucan administration. J Immunopharmacol 1986; 8: 407-425.

[32] Williams DL, Sherwood ER, Browder IW, McNamee RB, Jones EL, Rakinic J and Di Luzio NR. Effect of glucan on neutrophil dynamics and immune function in *Escherichia coli* peritonitis. J Surg Res 1988; 44: 54-61.

[33] Jorgensen JB, Lunde H and Robertsen B. Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, *Salmo salar* L. J Fish Dis 1993; 16: 313-325.

[34] LeBlanc BW, Albina JE and Reichner JS. The effect of PGG-beta-glucan on neutrophil chemotaxis in vivo. J Leukoc Biol 2006; 79: 667-675.

[35] Brown GD and Gordon S. Immune recognition of fungal beta-glucans. Cell Microbiol 2005; 7: 471-479.

[36] Engstad CS, Engstad RE, Olsen JO and Osterud B. The effect of soluble beta-1,3-glucan and lipopolysaccharide on cytokine production and coagulation activation in whole blood. Int Immunopharmacol 2002; 2: 1585-1597.

[37] Nakagawa Y, Ohno N and Murai T. Suppression by *Candida albicans* beta-glucan of cytokine release from activated human monocytes and from T cells in the presence of monocytes. J Infect Dis 2003; 187: 710-713.

[38] Toklu HZ, Sener G, Jahovic N, Uslu B, Arbak S and Yegen BC. Beta-glucan protects against burn-induced oxidative organ damage in rats. Int Immunopharmacol 2006; 6: 156-169.

[39] Saeij JP, Stet RJ, de Vries BJ, van Muiswinkel WB and Wiegertjes GF. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev Comp Immunol 2003; 27: 29-41.

[40] Savan R and Sakai M. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L.): genomic and expression analysis. Fish Shellfish Immunol 2004; 17: 87-94.

[41] Zou J, Wang T, Hirono I, Aoki T, Inawaga H, Honda T, Soma GI, Ototake M, Nakanishi T, Ellis AE and Secombes CJ. Differential expression of two tumor

necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Dev Comp Immunol 2002; 26: 161-172.

[42] Savan R, Kono T, Igawa D and Sakai M. A novel tumor necrosis factor (TNF) gene present in tandem with the TNF-α gene on the same chromosome in teleosts. Immunogenetics 2005; 57: 140-150.

[43] Igawa D, Sakai M and Savan R. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and IL-26 genes have been described for the first time outside mammals. Mol Immunol 2006; 43: 999-1009.

[44] Bogdan C, Mattner J and Schleicher U. The role of type I interferons in non-viral infections. Immunol Rev 2004; 202: 33-48.

[45] Ren Y and Savill J. Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J Immunol 1995; 154: 2366-2374.

[46] Laing KJ and Secombes CJ. Chemokines. Dev Comp Immunol 2004; 28: 443-460.

[47] Sangrador-Vegas A, Lennington JB and Smith TJ. Molecular cloning of an IL-8-like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression subtractive hybridization. Cytokine 2002; 17: 66-70.

[48] Laing KJ, Cunningham C and Secombes CJ. Genes for three different isoforms of transforming growth factor-beta are present in plaice (*Pleuronectes platessa*) DNA. Fish Shellfish Immunol 2000; 10: 261-271.

Figure legends

Figure 1: A) Cumulative mortality percentage at 96 h postchallenge in adult zebrafish ip infected with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml that 6, 4 and 2 days prior to challenge were inoculated with 10 µl of PBS or 0.5, 2 and 5 mg/ml of β-glucan. B) Cumulative mortality percentage at 96 h post-challenge in adult zebrafish ip infected with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml that 6, 4 or 2 days prior to challenge (dbc) were inoculated with 10 µl of PBS or 5 mg/ml of β-glucan. Each bar represents the mean of two duplicates. Error bars represents standard deviation.

Figure 2: A) Percentage of myelomocytic cells measured by flow cytometry from kidney of zebrafish challenged with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml for 6h and that were inoculated with 10 µl of PBS or β-glucan 5 mg/ml at 6, 4 and 2 days prior to challenge. Statistical difference between infected and mock infected zebrafish is given by †($P<0.05$). Statistical difference from control group (PBS pretreated) is given by ** ($P<0.01$). B) Bactericidal activity in kidney cells. Statistical difference from control group (PBS pretreated) is given by ** ($P<0.01$).

Figure 3: Proinflammatory cytokines and chemokines expression levels in kidney cells from zebrafish challenged with 10 µl of *A. hydrophila* at a dose of 10^8 cfu/ml or mock infected with PBS for 30 min, 2, 4 and 6h and that were inoculated with 10 µl of PBS or β-glucan 5 mg/ml at 6, 4 and 2 days prior to challenge. Each point represents the mean of 3 pools (3 zebrafish/pool) for proinflammatory cytokines and 2 pools (3 zebrafish/pool) for chemokines. Error bars represent standard deviation.

Table legends

Table 1: Sequences of oligonucleotide primers of CXCL-C1c, CC-chem and IL8.

Reviewers' comments:

Reviewer #1:

1. Author should describe zebrafish size and weight.

The zebrafish size and weight is now described in line 73.

2. How much volume inject to zebrafish and also what kind of needle did you use?

The injected volume was added in several sentences and the type of needle used is described in line 82.

3. In material and methods section the author should write about the expression analysis of TNF alpha, IL1beta, IFN gama (conditions of the real-time PCR)

This has been corrected (lines 115-130)

4. In the results section the author should show the figure results of the flow cytometry analysis of the myelomonocytic cells population.

We believe that Figure 2A is enough to explain the results showing the percentages of the myelomonocytic cells population, because it clearly shows the effect of glucans and bacteria in the percentage of the myelomonocytic cells.

5. Page 4 lines 68, 92 and 94 <<myelomonocityc>> should be myelomonocytic

This has been corrected.

6. Page 15: figure 1 (A and B) and Page 16: figure 2C the author should change the white figure to other color.

This has been changed.

7. Two copies of TNF have been cloned in rainbow trout and carp, which have been named TNF-1alpha and TNF-2alpha (refer to Savan and Sakai, 2004). The author should make a comparison with TNF-1alpha and TNF-2alpha published.

The existence of different copies of TNF was commented (lines 198-204).

8. The author should compare the result of IFN gamma in the study with the two interferon (IFN) like gamma that has been reported refer to Savan and Sakai, 2006).

It was also commented (lines 205-210).

9. The author should do expression analysis of chemotactic cytokines such as chemochines (CC and CXC) (refer to Laing and Secombes, 2004a) and other cytokines implicated in inflammation for example IL-18.

Additional experiments were conducted. The expression analysis of some chemokines was performed (CXCL-C1c, CC, IL8). This has been included in Material and Methods (lines 121-122) and also in Results and Discussion (lines 226-244).

Reviewer #2:

1. Line 25: Intraperitoneal injection of 5 mg/ml of <beta>-glucan. As long as the authors don´t state the volume of the injection it makes no sense. This lack of exact statement reduces the quality of the manuscript.

This has been corrected.

2. The <beta>-glucan was injected intraperitoneally and the fish were shallenged by intraperitoneal injection of A. hydrophila. A better challenge-model is to challenge the fish by immersion (bathing) which is a more natural way of introducing infection to the fish.

The challenge immersion was not conducted because it does not cause mortalities unless induce a wound on the fish. (Rodríguez et al., Fish Shellfish Immunol 2008; 25: 239-249.)

3. Below are listed line numbers that have to be amended with regard to statement of dose and not just concentration:
Lines: 23, 25, 26, 81/82, 82, 85, 87, 89, 96, 109, 123, 126, and legends to figures 1 and 2.

This has been corrected.

4. Minor things.

All the minor changes indicated by the referee have been corrected.

Figure(s)

Figure 1

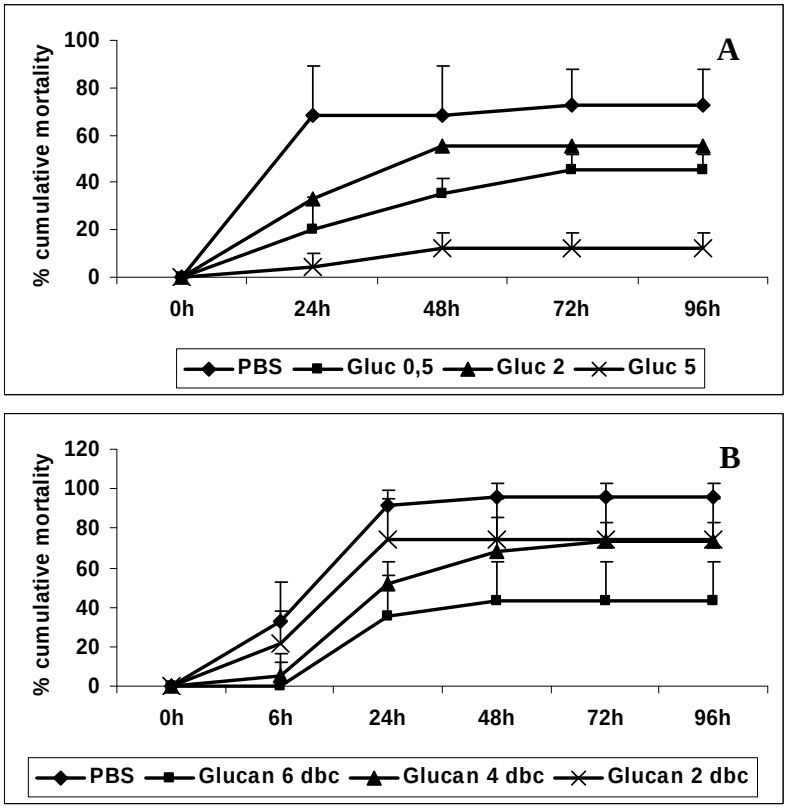

Figure 2

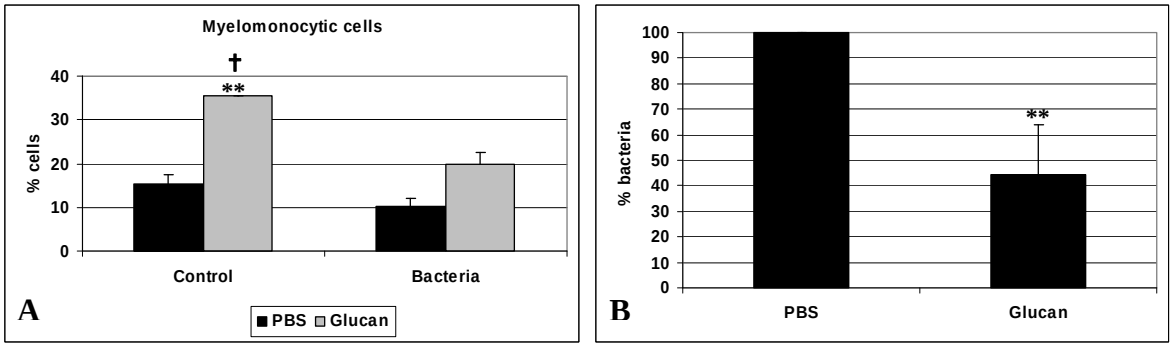

Figure 3

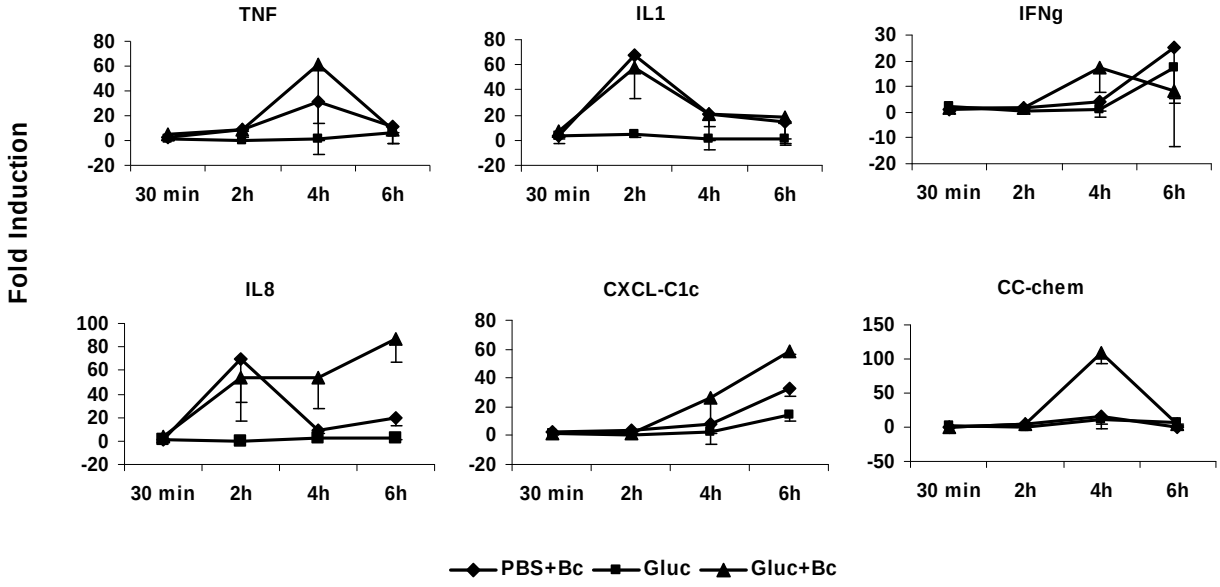

Table 1

	Sequence Primers 5´-3´		Nº accession GenBank
	Forward	Reverse	
CXCL-C1c	CTGCTGCTTGCGGTAGTTTA	TCAACTTTGTCGCAGTTTGG	NM_001115060
CC-chem	TGCAGCTCAACCAGAAGATG	CTTTGACGCATGGAGGATTT	BC162421.1
IL8	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG	XM_001342570.2